

- 1 -

TRANSGENIC ANIMALS HAVING A MODIFIED GLYCOPROTEIN V GENE

Inventors: **Vanitha Ramakrishnan and David Philips**

5

CROSS REFERENCES TO RELATED APPLICATIONS

This application claims priority to United States provisional patent application Serial No. 60/109,797, filed August 4, 1998, the disclosure of which is hereby incorporated by reference in its entirety.

10

FIELD OF THE INVENTION

The present invention relates to nonhuman, transgenic animals in which the Glycoprotein V (GP V) gene has been modified so that the animals do not express a functional GP V protein or express a GP V protein that demonstrates a reduced functionality as compared with the native or wild-type GP V protein. Such modifications may be accomplished, for example, through inactivation of the GP V gene by deletion or through targeted disruption of its genomic coding region. In particular, the present invention relates to a nonhuman, transgenic mammal, such as a mouse or rat, that does not express a GP V protein or that expresses a modified GP V protein. The present invention also relates to transgenic animal model systems and to transgenic, isolated cell model systems useful to identify agents that modulate the effects of such a GP V modification. In particular, the isolated cell model system relates to platelets isolated from such transgenic animals.

25

BACKGROUND OF THE INVENTION

The GPIb-IX-V complex is a large multimeric protein complex on the platelet surface which consists of 4 different subunits GPIb α , GPIb β , GPIX and GP V in the ratio of 2:2:2:1. Absence of some or all of the subunits of this complex results in a severe recessive bleeding disorder known as Bernard-Soulier syndrome (Blood (1998) 91(12):4397-4418). The GPIb α subunit contains the high affinity binding site for thrombin and the binding site for vWF. This complex has been implicated in the initial events associated with arterial thrombosis (Savage *et al.* (1996) Cell 84:289-297 and Weiss (1995) Thrombosis and Haemostasis 74(1):117-122).

It is known that GP V is a platelet and endothelial cell specific glycoprotein, and that it is a substrate for thrombin. It also is known that the activation of platelets by thrombin results in the loss of surface GP V. However, the precise role that GP V plays in the function of the GPIb-IX-V complex has not been described.

SUMMARY OF THE INVENTION

The present invention provides nonhuman transgenic animals, preferably mammals, that contain or comprise a modified GP V gene. The genomic GP V gene of such transgenic animals has been modified in the sense that it has been deleted, in whole or in part, or that it has been altered, substituted or mutated in some way. The particular modification is not critical so long as the cells of the transgenic animal do not express a GP V protein, do not express a functional GP V protein or express a GP V protein that demonstrates a modified (*i.e.*, reduced) functionality as compared with the same type of cell that expresses the native or wild-type GP V protein. Examples of mammals contemplated by the present invention include sheep, goats, mice, pigs, dogs, cats, monkeys, chimpanzees, hamsters, rats, rabbits, cows and guinea pigs.

DRAFTED
RECORDED
SEARCHED
INDEXED
FILED

- 3 -

The present invention also provides cells isolated from such animals, including platelets and other blood cells isolated from the blood of the transgenic non-human animals according to the present invention.

5 The present invention also provides methods of preparing a nonhuman transgenic animal, preferably a mammal, with a modified GP V gene. Such modification may be accomplished by techniques that are known in the art and that are discussed below.

10 The present invention also provides methods of comparing a characteristic between two mammals of the same species, or strain, wherein one mammal has, for example, a wild-type GP V gene and the other mammal has an modified GP V gene. The present invention similarly provides methods for comparing cells isolated from such mammals.

15 The present invention also provides methods of determining the effect of various agents on selected biological characteristics of a genetically engineered animal expressing a modified GP V gene, wherein the methods comprise: a) administering the agent to the genetically engineered animal; b) maintaining the animal for a desired period of time after administration of the agent; and c) determining whether a characteristic of the animal that is attributable to the expression of the modified GP V gene has been affected by the administration of said agent. The present invention similarly provides methods for determining the effects of various agents on the phenotypical, physiological, or biological 20 characteristics of cells isolated from such genetically engineered animals.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the murine DNA sequence of GP V.

25

Figure 2 shows the murine GP V amino acid sequence.

- 4 -

Figure 3 shows the human DNA sequence of GP V.

Figure 4 shows the human GP V amino acid sequence.

5 Figure 5 shows the genomic organization of the mouse GP V gene and structure
of the targeting vector pGP VKO. Mouse GP V was mapped from a BAC ES129 library
and a 5' homology region (8Kb) consisting of the Xma1(blunt)-Bam H1 fragment was
cloned into the Acc 651(blunt)-BamHI sites on the vector pPN2T, and the 3' homology
region (1.4Kb) consisting of the XhoI-HindIII was cloned into the corresponding sites
10 around the Neo^r cassette, as shown.

15 Figure 6 shows binding of GP V -/- platelets to immobilized human vWf. Pooled
WP from wt (□), +/- (▽) and -/- (○) mice were incubated as described in Methods. The
data shown is the average of duplicates and is representative of 3 such experiments.

20 Figure 7 shows binding of FITC~vWf to GP V-/- platelets. Pooled PRP from GP
V wt (□), +/- (dashed) and -/- (■) mice were incubated with FITC~vWf and botrocetin
and analyzed by flow cytometry. The data is representative of 3 experiments done in
duplicate.

25 Figure 8 shows thrombin-induced FITC~fibrinogen binding in washed platelets
from GP V wt, +/- and -/- mice. Mouse WP from individual mice [wt (□) +/- (▽) and -/-
(○)] were stimulated with the indicated amounts of thrombin. The platelets were
incubated with FITC~labeled fibrinogen for 30 min fixed and analyzed by flow
cytometry. The data is representative of 3 experiments done in duplicate.

Figure 9 shows thrombin-induced aggregation in washed platelets from GP V wt, +/- and -/- mice. WP were prepared from 6-10 mice of each genotype, which were littermate controls. Platelet aggregation was determined in an aggregometer (Chrono-Log Corp). Five such experiments were carried out, and 4 out of five worked in the manner

5 shown.

DETAILED DESCRIPTION

The present invention relates to the production and use of nonhuman transgenic animals, preferably mammals, that contain or comprise a modified GP V gene.

10 Specifically, the genomic GP V gene of such transgenic animals has been modified in the sense that it has been deleted, in whole or in part, or that it has been altered, substituted or mutated in some way. Particularly contemplated are those modifications in which the cells of the transgenic animal do not express a functional GP V protein or express a GP V protein that demonstrates a reduced functionality as compared with the same type of cell that expresses the native or wild-type GP V protein. Thus, the nature of a particular modification is not critical so long as transgenic animal or transgenic cells contain or express such a modified GP V gene. The present invention also relates to cells isolated 15 from such transgenic animals. Particularly contemplated are platelets and other blood cells isolated from the blood of the transgenic non-human animals according to the 20 present invention.

Figure 1 provides the DNA sequence of the mouse GP V gene. In one aspect of the present invention, transgenic animals containing or expressing modified sequences of this GP V-encoding DNA sequence can be generated using knock-out procedures that are known in the art to disrupt the genomic gene. A variety of known procedures are 25 contemplated, such as targeted recombination. Once generated, such a transgenic or genetically-engineered animal, for example, a "knock-out mouse", can be used to

- 6 -

- 1) identify biological and pathological processes mediated by GP V; 2) identify proteins
and other genes that interact with the GP V protein; 3) identify agents that can be
exogenously supplied to overcome the absence or reduction in GP V protein function; and
4) serve as an appropriate screen for identifying agents that modulate (*i.e.*, increase or
5 decrease) the activity of the transgenic cells of knock-out mice or other animals so
modified.

In general, a transgenic nonhuman animal according to the present invention may
be prepared by producing a vector containing an appropriately modified GP V genomic
sequence and then transfecting such a vector into embryonic stem cells (ES Cells) of that
10 animal species. For example, in the production of a transgenic mouse according to the
present invention, transfected mouse ES Cells that had undergone a homologous
recombination event at the GP V locus were then identified by restriction analysis
Southern blotting. The desired modified ES cells were then injected into blastocysts in
order to generate chimeric mice which were bred to wild-type mice to produce
15 heterozygote animals expressing one normal and one modified GP V allele (as assessed
by Southern blotting of tail genomic DNA). Through conventional breeding techniques
thereafter, heterozygotic (or chimeric) females may then be crossed with chimeric males
to generate homozygotes.

Platelets from such transgenic mice can be used in a number of assays to identify
20 agents that modulate GP V function or to assess the role of GP V in platelet function. For
example, the bleeding time of modified or transgenic mice can be monitored in a
screening assay to identify agents that improve or restore the wild-type clotting
phenotype. Such assays also may help to elucidate the extent to which GP V is critical for
normal hemostasis.

25 In a preferred embodiment of the present invention, a mouse was generated in
which the GP V gene was modified by targeted disruption of the GP V coding region to

inactivate the GP V gene. The platelets from these animals may be expected to show certain phenotypic effects resulting from the loss of a fully-functional GP V gene (and expression product) on both the expression and function of the GPIb-IX-V complex. Specifically, the intact transgenic animals and cells derived from such animals may be used to evaluate the activity of various agents that modulate GP V function. Such animals and cells also provide a model system useful in evaluating the consequences of GP V cleavage on the function of the GPIb-IX-V complex and in identifying agents useful in modulating these consequences.

Unless defined otherwise, all technical and scientific terms used in this application have the same meaning as would commonly be understood by a person having an ordinary level of skill in the field to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described.

For example, the term "animal" is used herein to include all vertebrate animals including mammals, except humans. It also includes an individual animal in all stages of development, including embryonic and fetal stages. A "transgenic animal" is any animal containing one or more cells bearing genetic information altered or received, directly or indirectly, by deliberate genetic manipulation at a subcellular level, such as by targeted recombination or microinjection or infection with recombinant virus. The term "transgenic animal" is not intended to encompass an animal produced by classical cross-breeding alone or by *in vitro* fertilization alone, but rather is meant to encompass animals in which one or more cells are altered by or receive a recombinant, exogenous or cloned DNA molecule. This molecule may be specifically targeted to a defined genetic locus, be randomly integrated within a chromosome, or it may be extrachromosomally replicating DNA.

The term "knock-out" generally refers to mutant organisms, usually mice, which contain a null or non-functional allele of a specific gene. The term "knock-in" generally refers to mutant organisms, also usually mice, into which a gene has been inserted through homologous recombination. The knock-in gene may be a mutant form of a gene which replaces the endogenous, wild-type gene. Non-functional GP V genes include GP V genes which have been modified or inactivated, in whole or in part, by mutation or via any available method so that GP V expression is prevented, disrupted or altered so as to disrupt the wild type GP V phenotype. Such mutations include insertions of heterologous sequences, deletions, frame shift mutations and any other mutations that prevent, disrupt or alter GP V expression.

The transgenic mammals of the present invention therefore may display non-normal platelet aggregation and/or other effects. By comparing the physiological and morphological characteristics between the transformed and non-transformed animals, one skilled in the art can thereby determine the effect of the presence or absence of the GP V gene and its expression product on the corresponding animal.

The transgenic animals of the present invention can also be used to identify agents that modulate (*i.e.*, either promote or further inhibit) platelet aggregation or other effects that are mediated by the GPIb-IX-V complex. The evaluation of such agents can be conducted either *in vitro*, *in situ*, or *in vivo* by techniques known to those skilled in the art.

The cells, platelets, tissues and whole organisms of the disclosed transgenic animals specifically have utility in testing the effect of various agents for their ability to reduce or increase GPIb-IX-V complex mediated processes. Agents that can be tested include various anticoagulant, thrombolytic and antiplatelet therapeutics and drugs. Examples of such agents include glycosaminoglycans such as heparin; oral anticoagulants such as dicumarol, anisindione, and bromadiolone; tissue plasminogen activator (t-PA);

urokinase; aspirin; dipyridamole; and ticlopidine. See, Majerus, *et al.*, *Anticoagulant, Thrombolytic, and Antiplatelet Drugs*, in Goodman & Gilman's *The Pharmacological Basis of Therapeutics*, Ninth Edition, Chapter 54 (1996) for a more complete list of such agents and their pharmacology.

5 The cells and whole organisms of the transgenic animals of the present invention, quite apart from their uses in veterinary and human medicine, may also be used to investigate gene regulation, expression and organization in animals. In general, for further examples of diagnostic and research uses of transgenic mammals, especially transgenic mice, see U.S. Patent No. 5,569,824.

10

Homologous Recombination Techniques

Genes that are modified, truncated or replaced in whole or in part can be introduced into a target cell in a site directed fashion using homologous recombination. Similarly, homologous recombination techniques may be used to introduce a DNA sequence into the cells of an organism where a particular gene has been deleted from its native position in that sequence. Papers discussing homologous recombination are discussed in R. Kucherlapati *et al.*, (1995) U.S. Patent No. 5,413,923. Through these techniques, for example, a DNA sequence in which the GP V gene has been modified or deleted can be introduced. Such methods result in the creation of a transgenic animal, wherein the animal's genome has been modified, and the phenotype of the modified animal or cells from the modified animal can be studied for purposes of drug screening, investigating physiologic processes, developing new products and the like.

Homologous recombination permits site-specific modifications in endogenous genes and thus inherited or acquired mutations may be corrected, and/or novel alterations may be engineered into the target animal's genome. To prepare cells for homologous recombination in the generation of transgenic animals, embryonic stem cells or a stem cell

- 10 -

line may be obtained. Cells other than embryonic stem cells can be utilized (*e.g.*, hematopoietic stem cells, *etc.*) See, for more examples, J.G. Seidman *et al.*, (1994) U.S. Patent No. 5,589,369. The cells may be grown on an appropriate fibroblast fetal layer or grown in the presence of leukemia inhibiting factor (LIF) and then used. Once
5 transformed, the embryonic stem cells may be injected into a blastocyst that has been previously obtained, to provide a chimeric animal.

The main advantage of the embryonic stem cell technique is that the cells transfected with the "transgene" can be tested, prior to reimplantation into a female animal for gestation, to assess integration of the transgene and the effect of the transgene.

10 In contrast to the conventional microinjection technique, the homologous respective endogenous gene can be removed from a chromosome by homologous recombination with the transgene. By subsequent cross-breeding experiments, animals can be bred which carry the transgene on both chromosomes. If mutations are incorporated into the transgenes which block expression of the normal gene, the endogenous genes can be
15 eliminated by this technique and functional studies can thus be performed for purposes described above.

Homologous recombination can also proceed extrachromosomally, which may be of benefit when handling large gene sequences (*e.g.*, larger than 50 kb). Methods of performing extrachromosomal homologous recombination are described in R.M Kay *et*
20 *al.*, (1998) U.S. Patent No. 5,721,367.

Production of Transgenic Animals

Transgenic animals are genetically modified animals into which recombinant, exogenous or cloned genetic material has been experimentally transferred. Such genetic material is often referred to as a "transgene". The nucleic acid sequence of the transgene may be integrated either at a locus of a genome where that particular nucleic acid
25

TOKYO2000-E008137X260

sequence is not otherwise normally found or at the normal locus for the transgene. The transgene may consist of nucleic acid sequences derived from the genome of the same species or of a different species than the species of the target animal.

The term "germ cell line transgenic animal" refers to a transgenic animal in which
5 the genetic alteration or genetic information was introduced into a germ line cell, thereby
conferring the ability of the transgenic animal to transfer the genetic information to
offspring. If such offspring in fact possess some or all of that alteration or genetic
information, then they, too, are transgenic animals.

The alteration or genetic information may be foreign to the species of animal to
10 which the recipient belongs, or foreign only to the particular individual recipient, or may
be genetic information already possessed by the recipient. In the last case, the altered or
introduced gene may be expressed differently than the native gene.

The development of transgenic technology allows investigators to create animals
of virtually any genotype and to assess the consequences of introducing specific foreign
15 nucleic acid sequences on the physiological and morphological characteristics of the
transformed animals. In general, the availability of transgenic animals permits cellular
processes to be influenced and examined in a systematic and specific manner not
achievable with most other test systems. For example, the development of transgenic
animals provides biological and medical scientists with models that are useful in the study
20 of disease. Such animals are also useful for the testing and development of new
pharmaceutically active substances.

Transgenic animals can be produced by a variety of different methods including
transfection, electroporation, microinjection, gene targeting in embryonic stem cells and
recombinant viral and retroviral infection (see, e.g., U.S. Patent No. 4,736,866; U.S.
25 Patent No. 5,602,307; Mullins *et al.* (1993) Hypertension 22(4):630-633; Brenin *et al.*

(1997) *Surg. Oncol.* 6(2):99-110; Tuan (ed.), *Recombinant Gene Expression Protocols*, Methods in Molecular Biology No. 62, Humana Press (1997)).

A number of recombinant or transgenic mice have been produced, including those which express an activated oncogene sequence (U.S. Patent No. 4,736,866); express simian SV 40 T-antigen (U.S. Patent No. 5,728,915); lack the expression of interferon regulatory factor 1 (IRF-1) (U.S. Patent No. 5,731,490); exhibit dopaminergic dysfunction (U.S. Patent No. 5,723,719); express at least one human gene which participates in blood pressure control (U.S. Patent No. 5,731,489); display greater similarity to the conditions existing in naturally occurring Alzheimer's disease (U.S. Patent No. 5,720,936); have a reduced capacity to mediate cellular adhesion (U.S. Patent No. 5,602,307); possess a bovine growth hormone gene (Clutter *et al.* (1996) *Genetics* 143(4):1753-1760); and, are capable of generating a fully human antibody response (McCarthy (1997) *The Lancet* 349(9049):405).

While mice and rats remain the animals of choice for most transgenic experimentation, in some instances it is preferable or even necessary to use alternative animal species. Transgenic procedures have been successfully utilized in a variety of non-murine animals, including sheep, goats, pigs, dogs, cats, monkeys, chimpanzees, hamsters, rabbits, cows and guinea pigs (*see, e.g.*, Kim *et al.* (1997) *Mol. Reprod. Dev.* 46(4):515-526; Houdebine (1995) *Reprod. Nutr. Dev.* 35(6):609-617; Petters (1994) *Reprod. Fertil. Dev.* 6(5):643-645; Schnieke *et al.* (1997) *Science* 278(5346):2130-2133; and Amoah (1997) *J. Animal Science* 75(2):578-585).

The method of introduction of nucleic acid fragments into recombination competent mammalian cells can be by any method which favors co-transformation of multiple nucleic acid molecules. Detailed procedures for producing transgenic animals are readily available to one skilled in the art, including the disclosures in U.S. Patent No. 5,489,743 and U.S. Patent No. 5,602,307.

- 13 -

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the compositions of the present invention and practice the claimed methods. The following working examples therefore, specifically point out preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

5 Other generic configurations will be apparent to one skilled in the art.

METHODS

10 Proteins and Antibodies.

Rabbit antibodies against peptides based on mouse GP V generated by standard methods (Harlow, E. & Lane, D. (1988) *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor)) include Ab #810 (against mouse GP V residues C⁴⁷²-A⁴⁹⁰) and Ab#808 (against mouse GP V residues L⁴³²-R⁴⁵⁰). Anti-GP Ib-IX (rabbit polyclonal Ab #3584) was kindly provided by Dr. Beat Steiner, Hoffman-LaRoche (22). Anti- α IIb β 3 (Ab #41) was described previously (Law, D. A., Nannizzi-Alaimo, L., Ministri, K., Hughes, P. E., Forsyth, J., Turner, M., Shattil, S. J., Ginsberg, M. H., Tybulewicz, V. L. & Phillips, D. R. (1999) *Blood* 93, 2645-52).

Human fibrinogen (Enzyme Research Labs) or human vWF (Haematologic technologies) were FITC-labeled in 0.1M NaHCO₃ (1mg/mL) using FITC~celite (Molecular Probes). Labeling conditions were designed to obtain F/P ratios between 1 and 4.

Snake venom peptide botrocetin was purified as described from the venom of *Bothrops jaracaca* (Andrews, R. K., Booth, W. J., Gorman, J. J., Castaldi, P. A. & Berndt, M. C. (1989) *Biochemistry* 28, 8317-26) and kindly provided by J. Rose. Human glycocalicin was purified from outdated platelets as described (Vicente, V., Kostel, P. J. & Ruggeri, Z. M.

(1988) *J Biol Chem* 263, 18473-9) by affinity chromatography using mouse anti-human glycocalicin (MAb 5A12; kindly provided by Dr. Burt Adelman).

Generation of the GP V *-/-* Mouse.

5 Rat platelet RNA was isolated using RNazol from fresh rat platelets and the GP V coding region (700bp) obtained by RT-PCR using degenerate primers based on the human GP V sequence (Lanza, F., Morales, M., de La Salle, C., Cazenave, J. P., Clemetson, K. J., Shimomura, T. & Phillips, D. R. (1993) *J Biol Chem* 268, 20801-7), was cloned into pCR2.1 (Invitrogen) and sequenced. The rat GP V insert was used to screen a mouse 129 BAC library (Genome Systems) 10 and 2 positive clones (11487 and 11488) were identified.

Genomic DNA was isolated and a detailed map of ~22Kb of the mouse GP V generated (Figure 5). The 5' XmaI fragment (11Kb) and the EcoR1 fragment (4Kb, containing the mouse GP V gene) were isolated from BAC plasmid DNA, subcloned into BlueScript (Stratagene), and sequenced. The mouse 129 GP V coding sequence showed 91% homology to the rat coding 15 sequence, compared to 71% to human GP V.

The ~8Kb XmaI(blunt)-BamHI fragment from the XmaI plasmid was first subcloned into the vector pPN2T (Tybulewicz, V. L., Crawford, C. E., Jackson, P. K., Bronson, R. T. & Mulligan, R. C. (1991) *Cell* 65, 1153-63; Morrison, J. R., Paszty, C., Stevens, M. E., Hughes, S. D., Forte, T., Scott, J. & Rubin, E. M. (1996) *Proc Natl Acad Sci U S A* 93, 7154-9) at the Acc651(blunt)- 20 BamHI sites to generate the 5' homology region (HR) downstream of the Neo^r cassette, followed by the 1.4Kb XhoI-HindIII fragment from the EcoRI plasmid generating the 3' HR upstream of the Neo^r cassette. Thus in the recombinant, the coding region of mouse GP V (including the putative initiator Met) to Leu³⁸⁹ was replaced by a Neo^r cassette oriented transcriptionally in the opposite direction (Figure 5). pGP VKO was electroporated into the ES cell line RW4 (Hug, B. A., 25 Wesselschmidt, R. L., Fiering, S., Bender, M. A., Epner, E., Groudine, M. & Ley, T. J. (1996) *Mol Cell Biol* 16, 2906-12.).

PCT/US99/17594 2/60

- 15 -

Neo^r clones were identified by positive selection in G418 media. Recombinants were microinjected into embryos from C57BL/6J mice using standard techniques (Dr. R. Wesselschmidt, Genome Systems). One of several chimeric males generated was bred with C57BL/6J females. Confirmation of recombination and germline transmission were performed
5 using a probe designed to show linkage following SphI digestion of genomic DNA (10µg) isolated as described (Laird, P. W., Zijderveld, A., Linders, K., Rudnicki, M. A., Jaenisch, R. & Berns, A. (1991) *Nucleic Acids Res* 19, 4293) and Southern analysis. Heterozygote (+/-) animals identified by Southern blotting were bred to homozygosity.

10 **Mouse Platelet Preparation.**

Blood from anaesthetized mice was obtained by cardiac puncture diluted into saline containing 1/10 vol of either (a) TSC buffer (3.8% trisodium citrate, 0.111M glucose, pH7, 0.4µM prostaglandin E1 (PGE1)) for platelet-rich plasma (PRP) or (b) Acid-Citrate-Dextrose (ACD, 85mM sodium citrate, 0.111M glucose, 714mM citric acid, 0.4µM PGE1), for washed platelets (WP). Diluted blood was centrifuged at 82xg for 10 min.

The supernatant from (a) normalized to 2x10⁸platelets/mL and 1mM Mg²⁺ (final) was PRP. For WP, the supernatant from (b) was pooled with a second obtained by centrifugation after the repeat addition of 137mM NaCl, and centrifuged at 325xg for 10 min.

Platelets were washed twice in CGS buffer (12.9mM sodium citrate, 33.33mM glucose, 20 and 123.2mM NaCl, pH7) and resuspended in calcium-free Tyrodes-Hepes buffer (CFTH;10mM Hepes, 5.56mM glucose, 137mM NaCl, 12mM NaHCO₃, 2.7mM KCl, 0.36mM NaH₂PO₄, 1mM MgCl₂, pH7.4). Platelets were normalized to 2x10⁸/ml. PRP or WP were incubated at room temp for 30 min prior to use.

- 16 -

Glycoprotein Expression.

Flow cytometry was carried out as follows: PRP (10 μ l) was incubated with primary rabbit antibody in CFTH containing 0.1% BSA for 1 hour at 4°C, followed by phycoerythrin (PE)-conjugated donkey anti-rabbit IgG (H+L) F(ab')₂ (1:200, Jackson ImmunoResearch) for 30 min at 5 4°C in the dark. Samples were diluted to 400 μ L in PBS containing 0.1% BSA and analyzed on a

FACSSORT (Becton-Dickinson).

Western analysis was carried out as follows: WP (5x10⁷) were solubilized in reducing sample buffer (Laemmli, U. K. (1970) *Nature* 227, 680-5), electrophoresed, transferred to membranes and probed with primary rabbit antibody overnight at 4°C. Blots were incubated with peroxidase conjugated mouse anti-rabbit secondary antibody (1:5000; Jackson ImmunoResearch) for 1 hour at 4°C and developed by ECL (Amersham). 10

FITC~Ligand Binding Assay.

For the determination of solution-phase vWF binding, pooled PRP was incubated with FITC~vWF and botrocetin (4 μ M-40 μ M) for 10min. Samples were diluted in CFTH buffer prior to analysis. For FITC~fibrinogen binding, WP were isolated from individual mice and incubated in duplicate (1x10⁶) α -thrombin for 10 min. The reaction was terminated with PPACK (phenylalaninylproylargininylchloromethylketone; 50 μ M final). The platelets were incubated with FITC~labeled fibrinogen (100 μ gs/ml) for 30 min, fixed with *p*-formaldehyde (10%) for 20 min and diluted into 1% *p*-formaldehyde in CFTH and analyzed by flow cytometry. 20

Binding Assays.

96-well plates were coated with various concentrations (25-500 ng/well) of human vWF overnight at 4°C. Pooled WP from mice were resuspended in Mg²⁺-free CFTH buffer 25 (1.2x10⁸/mL) containing botrocetin (4 μ M), and incubated immobilized human vWF for 1hour at

TOKYO 2003 SEP 22 2000

- 17 -

room temp. Following two PBS washes, bound platelets were lysed and intracellular acid phosphatase activity was quantitated colorimetrically using the substrate pNPP (Sigma).

Platelet Aggregation.

5 Each experiment used pooled WP from 6-10 littermate mice of each genotype. Platelet aggregation initiated by thrombin was measured in a lumi-aggregometer (Chrono-Log) with stirring (1000-1200rpm) at 37°C.

Determination of Bleeding Time.

10 Bleeding time measurements were obtained using the tail cut model (Hodivala-Dilke, K. M., McHugh, K. P., Tsakiris, D. A., Rayburn, H., Crowley, D., Ullman-Cullere, M., Ross, F. P., Coller, B. S., Teitelbaum, S. & Hynes, R. O. (1999) *J Clin Invest* **103**, 229-38) on littermate mice generated from heterozygous breeding. Since complete litters were not used the numbers of wt to +/- and -/- do not reflect Mendelian ratios. All experiments were blinded.

15 Briefly, anaesthetized mice were transected at the 5mm mark from the tip of tail and incubated in warm (37°C) saline. The time for cessation of bleeding was noted as the primary endpoint. If bleeding did not stop in 15 min, the tail was cauterized and 900 sec noted as the bleeding time. Data are presented as mean ± sem, and statistical significance was assessed using both Student's t-test analysis and Mann-Whitney nonparametric analysis.

20

EXAMPLES

Example 1. Generation of the Targeting Vector.

1. Obtaining the murine GP V genomic DNA

25 The sequence of murine GP V was unknown at the start of this project. We therefore generated degenerate primers based on the human GP V sequence which had been published (Lanza et al (1993), J. Biol. Chem., Vol 268 (28) pp20801-20807, U.S. Patent Application No.

- 18 -

08/089,455, filed July 9, 1993, which is incorporated by reference herein and Figure 3). These primers had the following sequences:

Coding sequences:

5' GG5ATGAC5GT5(CT)T5CA(GA)CG 3' (SEQ ID NO: 1) which corresponds to human GP V residues GMTVLQR (SEQ ID NO: 2).

5' GA(CT)AA(AG)ATGGT5(CT)T5(CT)T5GA(GA)CA 3' (SEQ ID NO: 3) corresponds to human GP V residues DKMVLLEQ (SEQ ID NO: 4).

10

5' CC(CT)GG(CGA)AC(AC)TT(TC)AA(CT)GA(CT)(CT)T5GT(GC)AA 3' (SEQ ID NO: 5) corresponds to human GP V residues PGTFSDLIK (SEQ ID NO: 6).

Non-coding Sequences:

15

5' (AG)TT(TGC)C(TG)(AG)AA(AG)GC(AG)GC(AG)GG 3'(SEQ ID NO: 7) corresponds to human GP V noncoding sequence PAAAFRN (SEQ ID NO: 8)

5'GGCCA5A(AG)(TG)CC(AG)CA(AG)TC(AG)CA5A(AG)CCA(AG)GG 3' (SEQ ID NO: 9) corresponds to human noncoding SWRCDCGLG (SEQ ID NO: 10).

20

Fresh rat platelets were isolated by standard techniques and RNA was isolated using RNAzol. PolyA⁺ RNA was generated using the Oligo-Tex system. cDNA was prepared from the polyA⁺ RNA using the In-Vitrogen cDNA cycle kit. The cDNA was then used in PCR reactions with each combination of the primers listed above.

25

All PCR reaction products were then cloned into pCR2.1 cloning vectors from the In-Vitrogen TA cloning kit. GIBCO SURE competent cells were transformed using the

manufacturer's protocol and white (transformant) colonies were selected. Miniprep DNA was generated by the rapid boiling method and restriction analysis was used to identify the clones containing inserts of the right size (~700bp). Several clones were expanded for sequencing.

5 Sequence analysis showed that clone B1-12 was homologous to the human GP V gene, and was the rat homologue of the GP V gene.

The insert from this clone was isolated and used to screen the mouse 129 BAC library (Genome Systems) by hybridization (see Shizuya *et al.* (1992) Proceed. Nat'l. Acad. Sci. USA 89:8794-8797). 2 clones 11487 and 11488 were positive. Genomic DNA was isolated from these clones. Approximately ~22Kb of the insert was mapped using Southern blotting with the B1-12 insert. The mouse genomic DNA for GP V was identified by homology to the published human GP V DNA sequence (Figures 1 and 2).

10 2. Construction of the Targeting Vector.

The vector pPN2T (10.15Kb) is a modified version of the pPNT vector (Tybulewicz *et al* 15 (1991) Cell 65:1153-1163, Morrison *et al* (1996) Proc. Natl. Acad. Sci. USA 93: 7154-7159) which, in addition to the Neo resistance (Neo^r) cassette, has 2 contiguous herpes simplex virus thymidine kinase (TK) cassettes (instead of the single one in pPNT) and a pUC vector backbone. BAC plasmid DNA was isolated from clone 11488 using protocols supplied by Genome Systems. We isolated a 11-16Kb Xma1 fragment and a ~4Kb EcoR1 fragment which were subcloned into 20 BlueScript. The Xma1 containing plasmid was used to generate a ~8Kb Xma1-BamH1 fragment which was blunted using Klenow at the Xma1 site. This fragment was then subcloned into the targeting vector pPN2T at the Acc651(blunt)-BamH1 sites in the polylinker which was at the 3' end of the Neo^r cassette between the Neo^r and TK cassettes , such that its orientation was opposite to that of the Neo gene. The 1.4Kb Xho1-HindIII 3' homology region was isolated from 25 the BAC plasmid DNA using the same methodology, and subcloned into BlueScript and the cut out using Xho1-Not1. This Xho1-Not1 fragment was then inserted into the targeting vector at the

- 20 -

5' end of the Neo' cassette. The final targeting vector pGP VKO had the mouse GP V homology regions in the opposite orientation from that of the Neo gene.

5 **Example 2. Generation of ES Cells.**

The targeting vector was inserted into the ES cell line RW4 by electroporation by standard techniques (Genome Systems® and Hug *et al.* (1996) Mol. Cell. Biol. 16(6):2906-2912). Neo' clones were identified by positive selection in G418 media. Identification of the targeted ES cells which had undergone recombination was done using the restriction enzyme Sph 1 to digest the genomic DNA from the clones and Southern blotting with a probe designed to show linkage (outside probe).

10 Clone 367 was shown to be recombinant since Southern blot analysis showed the expected 2 bands, one at 6Kb (wild type allele) and one at 2Kb (recombinant) and was then micro-injected into embryos from C57Bl6 mice using standard techniques. Several chimeric males were 15 generated which were then bred with C57Bl6 females to determine germline transmission.

Example 3. Generation of GP V Knock-out mice.

To evaluate the specific role of GP V in both platelet function and the GP Ib-IX-V complex expression, we generated a mouse strain that lacked the GP V gene using homologous 20 recombination techniques (Koller, B. H. & Smithies, O. (1992) *Annu Rev Immunol* 10, 705-30). Since rat GP V RNA would have greater homology to mouse GP V than the cloned human gene and was easier to isolate than mouse platelet RNA, rat platelet RNA for GP V was isolated and used as a probe for isolating genomic mouse GP V which was mapped and cloned from a BAC 129/Sv library.

25 Complete sequencing of 3 separate clones showed the mouse 129/Sv GP V gene to have 99.9% homology in the coding region to the published mouse C57BL/6J mouse sequence

- 21 -

(Ravanat, C., Morales, M., Azorsa, D. O., Moog, S., Schuhler, S., Grunert, P., Loew, D., Van Dorsselaer, A., Cazenave, J. P. & Lanza, F. (1997) *Blood* 89, 3253-62) at both the DNA and protein levels (not shown). Sequencing also proved that the mouse and rat coding sequences were more homologous (DNA=92%, protein=87%), than human and mouse GP V (DNA=78%, protein=70%).

Recombinants were selected based on Southern analysis using the probe shown in Figure 5 and two recombinants were used to generate the founder chimeras. One of these founder males (>85% chimeric) was successfully mated with C57BL/6J females to produce +/- offspring, which were bred to generate homozygotes. Deficiency in the GP V gene has not affected viability at birth as evidenced by findings that the litters have expected Mendelian ratios of -/- offspring (1:4) and that the GP V-/- animals are fertile with no gross observable defects.

Analysis of whole blood from GP V-deficient animals showed the platelets were normal in both number and size. Platelet counts in whole blood were within the normal range {wt males = $6.52 \times 10^8/\text{ml}$ (n=17) and females = $5.6 \times 10^8/\text{ml}$ (n=8); +/- males = $7.56 \times 10^8/\text{ml}$ (n=15) and females = $5.12 \times 10^8/\text{ml}$ (n=12); -/- males = $7.02 \times 10^8/\text{ml}$ (n=21) and females = $5.36 \times 10^8/\text{ml}$ (n=9)}. There was a statistically significant difference in platelet recovery from whole blood from wt animals (males = $74 \pm 22\%$; females = $82.4 \pm 13\%$) and -/- animals (males = $62 \pm 19.5\%$, p =0.05; females = $67 \pm 14\%$, p=0.03). +/- animals showed intermediate recovery numbers, which were not statistically different {males = $71 \pm 19\%$, p_(wt to +/-)=0.55 and p_(+/- to -/-)=0.14; and females = $78 \pm 12\%$, p_(wt to +/-)=0.46 and p_(+/- to -/-)=0.065 }.

Platelets were isolated from the -/- animals to confirm that gene deletion resulted in absence of GP V protein expression and analyzed for GP V expression using GP V antibodies. No GP V protein was detectable either on the intact platelet surface using FACS analysis or in total platelet lysates as determined by Western blotting.

Example 4. Effect of GP V gene deletion on GP Ib-IX expression and function.

GP V is usually expressed in platelets as a complex with GP Ib-IX (Meyer, S. C. & Fox, J. E. (1995) *J Biol Chem* 270, 14693-9; Modderman, P. W., Admiraal, L. G., Sonnenberg, A. & von dem Borne, A. E. (1992) *J Biol Chem* 267, 364-9).

5 We used two techniques to determine whether absence of GP V from the platelet surface affected the expression of the other subunits of the GP Ib-IX-V complex. FACS analysis using an antibody specific for GP Ib-IX showed similar levels of GP Ib-IX in all three genotypes (Mean relative fluorescence units (RFU) \pm sd for wt = 547 \pm 106; +/- = 391 \pm 65; -/- = 483 \pm 56 with p_(wt to -/-) = 0.42, p_(wt to +/-) = 0.11 and p_(+/- to -/-) = 0.14). Western blot analysis confirmed that similar levels of

10 the GP Ib and GP IX were also present in platelet lysates from all three genotypes.

Two assays were used to determine whether the GP Ib expressed on GP V -/- platelets was functional. One assay measured the adhesion of platelets to immobilized vWF that was activated by botrocetin to bind GP Ib. Figure 6 shows that GP V -/- platelets bound to immobilized, botrocetin-activated human vWF in a manner indistinguishable from wt platelets. Under these 15 conditions, the binding of vWF to platelets is mediated entirely by GP Ib, since purified human glycocalicin (a soluble, extracellular fragment of GP Ib α that contains the vWF binding domain), inhibited botrocetin-induced binding of platelets to vWF in a concentration-dependent manner (not shown). We also found that soluble, activated vWF bound identically to platelets from all three 20 genotypes in PRP (Figure 7). Again, botrocetin-induced vWF binding could be completely inhibited by purified human glycocalicin (not shown). Furthermore, stimulation of α IIb β 3 on platelets by ADP and epinephrine did not induce soluble vWF binding (Figure 7). Thus GP Ib-IX expressed in the GP V-/- platelets was functional.

Example 5. Effect of GP V gene deletion on thrombin-induced platelet function.

25 As shown in Figure 8, thrombin at low concentrations (0.5nM) induced significantly increased binding of FITC-fibrinogen in GP V -/- platelets compared to wt (Mean RFU \pm sd

wt=7±1.2, +/-=13.2±0.9 and -/- = 22±0.8). This difference persisted at 1nM thrombin (Mean RFU ± sd wt =11.5±6.8, +/- = 27.9±9.45 and -/- = 42±1.8). However, platelets from all genotypes were able to bind FITC-fibrinogen equivalently at high (20nM) thrombin concentrations (Mean RFU± sd wt= 66.5 ±8.6, +/- =76.1±11.3 and -/- =72 ±0.2). The apparent EC₅₀ values for thrombin were approximately 2nM for wt platelets and 0.7nM for the -/- platelets. P-Selectin expression was also greater in the GP V-/- platelets at low thrombin concentrations, compared to wt (not shown).

Consistent with the FITC-fibrinogen results, platelets lacking GP V exhibited an increased aggregation response to thrombin compared to wt platelets. Indeed, platelets from GP V -/- mice aggregated when treated with sub-threshold concentrations of thrombin (0.5nM) that did not induce a significant response in wt platelets (Figure 9).

As observed in the fibrinogen binding studies, platelets from GP V+/- heterozygous animals gave an intermediate response in the aggregation assays. We determined if this increased responsiveness was related to increased expression of αIIbβ3, using an antibody specific for the mouse fibrinogen receptor. The levels of αIIbβ3 were comparable on platelets from animals of all three genotypes by flow cytometry (Mean RFU wt =1554±386; +/- =1246±202; and -/- = 1435±77; p _(wt to -/-)= 0.65, p _(wt to +/-)= 0.31 and p _(+/- to -/-)= 0.24), and were also similar by Western blotting.

Example 6. Determination of bleeding time.

To determine the consequences of enhanced platelet function in GP V -/- mice, bleeding time measurements were performed using a tail cut model, which was previously shown to be platelet dependent (Hodivala-Dilke, K. M., McHugh, K. P., Tsakiris, D. A., Rayburn, H., Crowley, D., Ullman-Cullere, M., Ross, F. P., Coller, B. S., Teitelbaum, S. & Hynes, R. O. (1999) *J Clin Invest* 103, 229-38; Tsakiris, D. A., Scudder, L., Hodivala-Dilke, K., Hynes, R. O. & Coller, B. S. (1999) *Thrombosis and Haemostasis* 81, 177-188).

Consistent with the *in vitro* data, GP V^{-/-} mice had a statistically shorter bleeding time (Mean \pm sem=178 \pm 21sec) than wt littermate control mice (276 \pm 35 sec, Student's T-test p=0.016). The bleeding time in the +/- animals was intermediate (224 \pm 25 sec) but not statistically different from either wt or ^{-/-} mice. Furthermore, 70% of the ^{-/-} mice had bleeding times less than 120 sec, compared to 50% of the wt and +/- mice. Conversely, 21.6% of the wt mice had bleeding times greater than 500sec, compared to 9.5% in the +/- mice and 8.5% in the ^{-/-} mice. The difference in bleeding time is also statistically significant using non-parametric analysis (Mann-Whitney test p=0.046). Thus the increased aggregability of the platelets from GP V^{-/-} mice observed in *in vitro* assays translates into a shorter bleeding time *in vivo*.

10

Example 7. Additional Examples.

As explained herein, the mice produced according to this invention are useful in addressing the role of GP V, which is part of a complex on the platelet surface that is critical for high shear (arterial) platelet adhesion. Preliminary examination of platelets from these mice has already dispelled the hypothesis that GP V is required for the expression of the complex on the platelet surface, since animals deficient in the GP V gene express the remainder of the complex (subunits GPIb-IX) at levels grossly equivalent to the platelets of wild type animals as determined by flow cytometry.

Based on this information, GP V may have several biological functions. These include the following.

1. Role in platelet activation by thrombin.

a) Cleavage of GP V by thrombin may be essential for the activation of the platelet by the subsequent generation of an intracellular signal which results in the inside-out activation of GPIIb-IIIa. If cleavage is essential, mice in which GP V is absent from the platelet surface would have a severe bleeding diathesis similar to that observed in Bernard-Soulier patients.

b) GP V may function analogous to a brake with cleavage of GP V allowing

- 25 -

platelet activation via signaling through the GPIb-IX complex, and inside out activation of GPIIb-IIIa. Platelets which are deficient or lacking in GP V would be proaggregable. Thus platelets from GP V deficient mice may show a predilection to aggregation and a tendency to aggregate more quickly than wild type mouse platelets.

5 2. Role in coagulation. The kinetics of GP V cleavage reveals that GP V is cleaved at very low thrombin concentrations and suggests a possible role for GP V cleavage as part of the cascade of reactions that occurs during coagulation. While it is believed that platelet surfaces contribute to the rapid generation of thrombin which is the end product of the coagulation cascade, the various proteins on platelets which may be involved have not yet been identified. Since the 10 cleavage of GP V occurs at concentrations of thrombin much lower than that required to cleave other platelet surface proteins, GP V cleavage may be part of the process which occurs in arteries which culminates in the formation of a thrombus.

15 Activators of GP V cleavage may have a prothrombotic effect. Prevention of GP V cleavage may be a viable means of preventing arterial thrombosis especially under high shear conditions. Accordingly, inhibitors of GP V cleavage may be useful as drugs to prevent the clinical consequences of platelet activation like unstable angina and myocardial infarction.

20 The availability of mice, genetically lacking GP V will allow for the testing of these GP V functions. Platelets from these mice can be used in a variety of assays such as aggregation, agglutination, fibrinogen binding in response to a number of platelet agonists like thrombin, ADP, thromboxane A2, collagen etc. in order to characterize the effect of loss of GP V on the functional response of these platelets.

In addition the mice of Example 1 provide useful in vivo animal models of haemostasis and thrombosis which will allow for the determination of the role of GP V in such processes and should provide a specific model to study GP V as a therapeutic target.

- 26 -

Although the present invention has been described in detail with reference to examples above, it is understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims. All cited 5 patents, applications and publications referred to in the application are hereby incorporated by reference in their entirety.